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Precursor of the *myo*-inositol-binding protein of a *Pseudomonas* species

Dominique Feiss, Monique Belet and Jacques Deshusses*

Département de Biochimie, Université de Genève, 30 quai E. Ansermet, 1211 Genève 4, Switzerland

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The biosynthesis of the periplasmic binding protein of the *myo*-inositol high affinity transport system in a *Pseudomonas* strain involves the synthesis of a precursor of 2 kDa heavier than the mature form (30 kDa). The identification of the precursor is based on the comparison of the fingerprints of the two proteins and on kinetics in pulse-chase experiments.

Pseudomonas *Precursor protein* *Periplasmic protein* myo-*Inositol*

1. INTRODUCTION

Periplasmic binding proteins are synthesized in the cytoplasm of bacteria as precursors and subsequently exported with concomitant processing that involves the removal of a signal peptide [1]. The full sequence of the precursor and mature form of the arabinose-binding protein as well as the histidine-binding protein of the enterobacteria *E. coli* [2] and *Salmonella* [3] has been determined.

It was interesting to examine whether such a process also occurs in evolutionarily more distant bacteria such as soil *Pseudomonas*. In our laboratory we have previously reported that a periplasmic binding protein is involved in the active transport of *myo*-inositol in *Pseudomonas* sp. [4]. This binding protein has been isolated, characterized and partially sequenced [5]; its molecular mass is 30 kDa and it is devoid of cysteine.

Using pulse-chase experiments we have been able to show the formation of a precursor of 32 kDa. The confirmation that this protein is the precursor of the *myo*-inositol binding protein was obtained by the marked similarity that exists between the peptides obtained by partial digestion of the 32 kDa protein and the *myo*-inositol binding protein. Several inhibitors of the maturation process have been tested to increase the amount of precursor but only TAME showed a marked effect.

2. MATERIALS AND METHODS

2.1. *Strain and growth conditions*

The *Pseudomonas* species studied was isolated from soil by selective culture on *myo*-inositol in minimal medium [6]. The induced cells were grown at room temperature (20°C) in minimal medium supplemented with 1% *myo*-inositol.

2.2. *Chemicals*

[^{35}S]Methionine (approx. 1000 Ci/mmol) was purchased from the Radiochemical Centre (Amersham). TAME and procaine were obtained from Sigma (St. Louis, MO), phenethyl alcohol from Merck (Darmstadt, FRG) and Protein A-Sepharose from Pharmacia (Uppsala, Sweden). The other products, of the highest degree of puri-

* To whom correspondence should be addressed

Abbreviations: PAGE, polyacrylamide gel electrophoresis; PBS, phosphate saline buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH 7.4); TAME, *p*-tosyl-L-arginine methyl ester

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ty, were from Merck (Darmstadt, FRG) or Fluka (Buchs, Switzerland).

2.3. *Labelling and immunoprecipitation*

[^{35}S]Methionine labelling was performed as in [7] with the following modifications: pulses were 60–120 s at 20°C; the pellet obtained by trichloroacetic acid precipitation was washed twice with 10% trichloroacetic acid and 3 times with a mixture of 70% acetone/20% ethanol/10% Tris 100 mM (pH 8). The trichloroacetic acid precipitated material was resuspended in 500 μl PBS supplemented with 0.2% SDS, sonicated and centrifuged. Serum and 5 mg of Protein A-Sepharose in 50 μl PBS/0.5% Nonidet P40 were added to the supernatant and the samples were gently stirred overnight at 4°C (the amount of serum to be added was previously determined by immunodiffusion to bind all the antigen). The gel was washed 4 times for 1 h with PBS/0.5% Nonidet P40 and the immune complexes were eluted at 100°C for 20 min with 50 μl of electrophoresis buffer containing 2% SDS and analysed by SDS–PAGE [8].

2.4. *Fingerprints*

Limited hydrolysis of the proteins was performed as in [9] using V_8 *Staphylococcus aureus* protease. Proteolysis was carried out in the gel with 1 μg of protease per slot.

3. RESULTS

3.1. *Pulse-chase experiments*

Pulses of 60–120 s were chosen since they gave the optimal ratio of labelled precursor relative to mature protein. These pulses are much longer than those used with *E. coli* [7] because the growth temperature of this *Pseudomonas* sp. is lower (20°C), and its doubling time is about 6 times longer than that of *E. coli*. Fig.1 shows the densitometric pattern of a 10% SDS–PAGE representative of a pulse-chase experiment and immunoprecipitation. Two bands corresponding to 32 and 30 kDa are seen after the 120 s pulse and the 32 kDa band decreases upon increasing time of chase.

3.2. *Inhibitors*

Since procaine and phenethyl alcohol have been successfully used in *E. coli* as maturation inhibitors of periplasmic proteins, these substances were tested on *Pseudomonas* sp. The optimal conditions reported for *E. coli* are preincubation with 0.55% procaine [10] for 1 h and with 0.3 to 0.4% phenethyl alcohol [11] for 1 or 2 min. Using *Pseudomonas* sp. growing at 20°C, these inhibitors were tested over a wide concentration range to determine the optimal conditions. Procaine was added in concentrations varying from 0.025 to 0.5% with 30–120 min preincubation times and phenethyl alcohol from 0.2 to 0.5% with 2–5 min preincubation times. While various degrees of inhibition of the synthesis of the binding protein were observed depending upon the inhibitor concentration, no substantial increase in the 32 kDa protein was obtained (not shown).

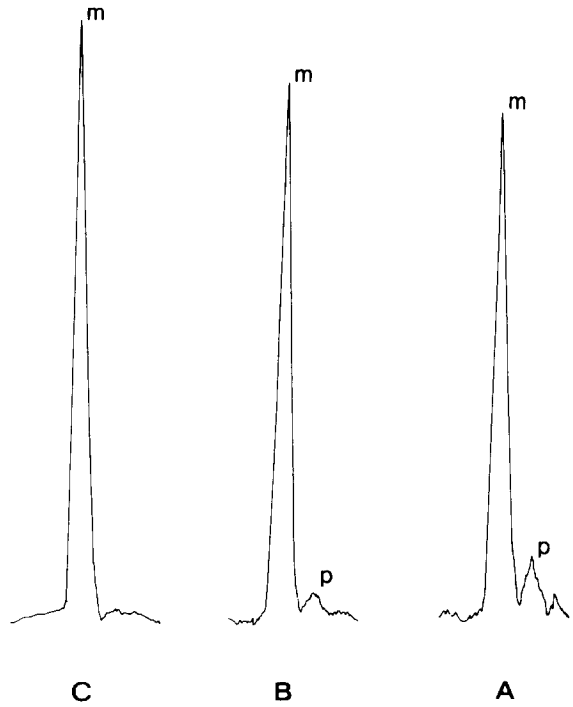

Fig.1. Densitometric tracing of a pulse-chase experiment. Pulse labelling and immunoprecipitation were performed as described in section 2. Pulse was 120 s. (A) No chase, (B) chase 30 s, (C) chase 120 s; p, precursor; m, mature protein.

3.3. *Reversible accumulation of precursor* myo-*inositol-binding protein in vivo*

The amount of the 32 kDa protein increased

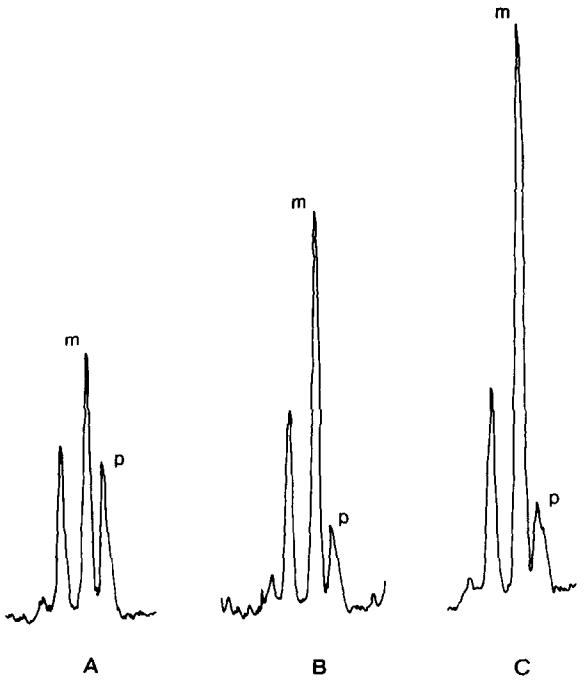

Fig.2. Processing of accumulated precursor. Densitometric tracing of an autoradiograph of material immunoprecipitated after labelling in the presence of TAME. Cells were preincubated for 5 min with 5 mM TAME. After a 2-min pulse with [^{35}S]methionine the cells were chilled on ice, centrifuged and resuspended in minimal medium containing 250 μg/ml methionine and 100 μg/ml chloramphenicol. (A) 30 s, (B) 2 min, (C) 5 min after resuspension. Symbols as in fig.1.

markedly relative to the mature form in the presence of TAME, a reversible maturation inhibitor utilized with *E. coli* [7]. Cells were preincubated for 5 min with 0.1–10 mM TAME. At 5 mM TAME, close to 50% of the immunoprecipitated material was in the precursor form 30 s after the removal of TAME and resuspension of the cells in the medium. From the ratio of the precursor to the mature form, one concludes that processing resumes. After 5 min essentially the mature form could be seen (fig.2).

3.4. *Fingerprints*

The radioactive bands of the mature and of the 32 kDa proteins were excised and submitted to partial digestion with V_8 *S. aureus* protease as in [8]. Fig.3 shows the densitometric pattern of the 15% SDS–PAGE demonstrating that the 32 kDa

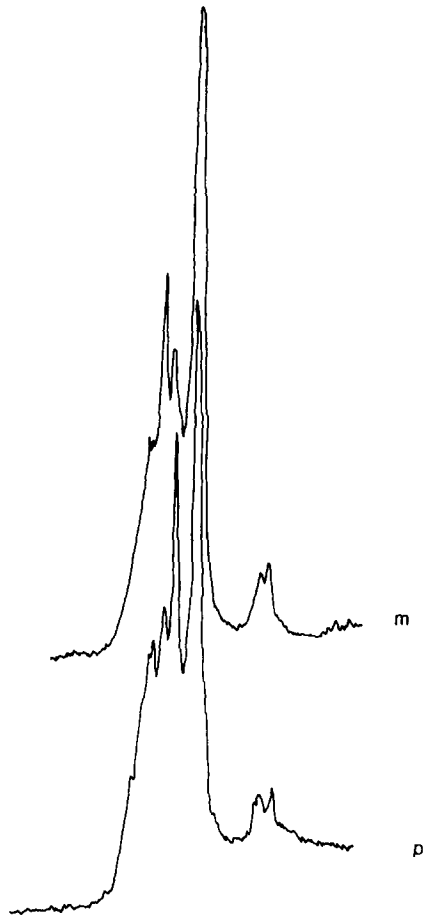

Fig.3. Densitometric tracing of limited proteolysis of mature protein (m, upper tracing) and precursor (p, lower tracing).

protein is the precursor of the mature *myo*-inositol-binding protein.

4. DISCUSSION

In enterobacteria it was shown that periplasmic proteins are synthesized as cytoplasmic precursors of higher molecular mass, and that processing involves the removal of a N-terminal segment. We chose to investigate whether such a process also occurs in soil organisms. We have isolated from soil a *Pseudomonas* sp. which is able to transport *myo*-inositol by a high affinity transport system that involves a periplasmic binding protein [4]. This *myo*-inositol binding protein was isolated and characterized [5]. In vivo, pulse-chase experiments

with [^{35}S]methionine followed by immunoprecipitation showed by SDS–PAGE in addition to the mature binding protein, a band which could correspond to a protein 2 kDa heavier than the binding protein (fig.1). Since this band faded upon chase with a corresponding increase of the mature protein, a direct relationship between these two proteins is obvious. Such kinetics are in agreement with other experiments showing precursor processing; e.g., in enterobacteria. The ultimate proof that this protein is the precursor of the binding protein comes from the pattern of peptides obtained by limited digestion. The densitometric tracings of the autoradiograms of SDS–PAGE patterns obtained after incubation of the mature protein or its presumed precursor with *S. aureus* V_8 protease show a very strong homology. This result is in favour of the postulated mechanism of biosynthesis of the *myo*-inositol binding protein which involves post-translational cleavage.

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